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Alternative G protein-coupling and biased agonism: new insights into melanocortin-4 receptor signalling

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Abbreviations: ACTH, adrenocorticotrophic hormone; cAMP, cyclic 3',5'-adenosine monophosphate; AGRP, agouti-related protein; CTX, *cholera* toxin; ER, endoplasmic reticulum; ERK-1/2, extracellular signal-regulated kinases; FSK, forskolin; GPCR, G protein-coupled receptors; GTP γ S³⁵, guanosine 5'-(3-O-thio)triphosphate; IRS, insulin receptor substrate; MGRN-1, mahogunin ring finger-1; MC4R, Melanocortin-4 receptor; MRAPs, melanocortin receptor accessory proteins; MSH, melanocyte-stimulating hormones; PKA, protein kinase A; PKC, protein kinase C; PI3K, phosphatidylinositol-3-kinase; PLC, phospholipase C; POMC, proopiomelanocortin; PTX, *pertussis* toxin; RAMPs, receptor-activity modifying proteins

Abstract

The melanocortin-4 receptor (MC4R) is a prototypical G protein-coupled receptor (GPCR) that plays a considerable role in controlling appetite and energy homeostasis. Signalling initiated by MC4R is orchestrated by multiple agonists, inverse agonism and by interactions with accessory proteins. The exact molecular events translating MC4R signalling into its physiological role, however, are not fully understood. This review is an attempt to summarize new aspects of MC4R signalling in the context of its recently discovered alternative G protein coupling, and to give a perspective on how future research could improve our knowledge about the intertwining molecular mechanisms that are responsible for the regulation of energy homeostasis by the melanocortin system.

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1 The melanocortin system

1.1 Agonists, receptors and physiological role

The melanocortin precursor molecule proopiomelanocortin (POMC) gives rise to at least 4 different melanocortins: the melanocyte-stimulating hormones (MSH) which are named α -, β - or γ -MSH and the adrenocorticotrophic hormone (ACTH) (Pritchard et al., 2003; Pritchard et al., 2002). Melanocortins are small peptide hormones that bind with different affinities to 5 distinct melanocortin receptors (MCR),

which belong to the super family of G protein-coupled receptors (GPCR) (Tab. 1). Melanocortins regulate pigmentation, adrenal hormone secretion, immune functions, lipid metabolism and feeding behaviours (Brzoska et al., 2008). The MC1R is expressed in many different tissues such as skeletal muscle, brain and lung, but its most established scene of action is the skin, where MC1R signalling is responsible for pigmentation (Suzuki et al., 1996). The MC2R is the receptor for ACTH and, thus, a strong stimulator of glucocorticoid production in vertebrates (Chida et al., 2007). MC3R and MC4R are abundantly found in the brain, where they

exert catabolic effects by decreasing food intake and increasing energy expenditure (Cone, 1999). Both MCR subtypes bind α - and β -MSH with similar affinity (Biebermann et al., 2006; Huszar et al., 1997; Lee et al., 2006), whereas, γ -MSH binds to the MC4R with a considerably lower affinity (by two orders of magnitude) compared to α -MSH (Voisey et al., 2003). Given the higher affinity of γ -MSH to the MC3R, it is assumed that this peptide induces its physiological effects mainly through the latter subtype. Interestingly, a newly discovered GPCR family, termed “mas-related GPCR” (Mrg), binds γ -MSH and its derivatives with similar high affinity, suggesting, that this peptide interacts with members of two unrelated GPCR families. The focus of γ -MSH research might turn to this novel aspect of melanocortin signalling in upcoming years (Han et al., 2002; Lembo et al., 2002).

The MC5R shows a broad expression pattern and targeted disruption of this gene results in widespread dysfunction of exocrine glands including a marked decrease in the production of sebum (Thiboutot et al., 2000). Since the complexity of signalling initiated by all 5 receptor subtypes and the melanocortins in different tissues is way beyond the scope of one review, in the following, we will concentrate on the MC4R.

The MC4R has been shown to play a pivotal role in controlling meal size and energy homeostasis. Adipose tissue-derived hormones such as leptin increase POMC expression in α -MSH-releasing neurons located in the arcuate nucleus of the hypothalamus (Shimizu et al., 2007). Secreted α -MSH activates MC4R expressing neurons of various hypothalamic nuclei, which, in turn, enhance the release of anorexigenic stimuli (e.g. thyrotropin- and corticotropin-releasing hormone) or inhibit the liberation of orexigenic peptides (e.g. orexins and melanin-concentrating hormone) (Ellacott and Cone, 2004).

The importance of MC4R signalling in the regulation of human metabolism has been highlighted by the finding that

mutations in the MC4R gene are the most frequent monogenic cause of severe obesity (Biebermann et al., 2003; Govaerts et al., 2005; Hinney et al., 2006; Hinney et al., 2003; Tao and Segaloff, 2003; Vaisse et al., 1998; Yeo et al., 2003). Accordingly, targeted disruption of the MC4R or the POMC gene in mice causes an obesity-diabetes syndrome characterized by hyperphagia, hyperinsulinemia and hyperglycemia (Balthasar et al., 2005; Huszar et al., 1997).

Besides its effects on the regulation of energy homeostasis, MC4R has also been shown to sensitise sexual sensation of males and females in humans (Hadley, 2005), to exhibit anti-inflammatory effects (Brzoska et al., 2008), to increase systolic blood pressure (Greenfield et al., 2009), to stimulate neurite elongation in DRG neurons after axonal injury (Tanabe et al., 2007) and to directly enhance allodynia (Starowicz et al., 2002).

1.2 Melanocortin-induced signalling pathways of the MC4R

Since the cloning of the MC4R by Gantz et al., in 1993, it has been established that this receptor subtype regulates intracellular concentrations of cyclic 3',5'-adenosine monophosphate (cAMP) by increasing adenylyl cyclase (AC) activity through G_s proteins and afterwards enhances activity of protein kinase A (PKA) (Gantz et al., 1993). Further, it has been reported that ligand-induced activation of the MC4R modifies the activity of extracellular-regulated kinases (ERK-1/2) (Chai et al., 2006; Patten et al., 2007; Vongs et al., 2004), AMP-activated kinase (Minokoshi et al., 2004), c-jun kinase (Chai et al., 2009), phosphatidylinositol-3-kinase (PI3K) (Vongs et al., 2004) and protein kinase C (PKC) (Chai et al., 2006). Down-stream of these kinases, it has been shown that MC4R signalling regulates ion channel activity (Fu and van den Pol, 2008), gene expression (Lee et al., 2001) and enhances insulin signalling by decreasing insulin receptor

substrate-1 (IRS-1) phosphorylation (Chai et al., 2009) (Fig. 1).

1.3 Alternative G protein coupling and signalling of the MC4R

Numerous studies clearly indicated that the MC4R interacts with G_s proteins in endogenous or recombinant expression systems. The importance of the G_s /PKA pathway has been shown by targeted disruption of the $R_{II\beta}$ -subunit of PKA in mice (Czyzyk et al., 2008). This work demonstrates that disruption of PKA activity alters adiposity and locomotor activity downstream of the MC4R in the hypothalamus. Interestingly, when recombinantly expressed, adipositas-associated MC4R mutants exerted inconsistent effects on G_s signalling. Some mutants showed a full loss-of-function phenotype, whereas others showed no functional alterations on G_s signalling. Even more surprisingly, some mutants constitutively increased adenylyl cyclase activity (Hinney et al., 2003). Therefore, no clear correlation could be drawn between the cellular phenotype resulting from these mutations and obesity observed *in vivo*. Thus, one might suggest that signalling pathways other than G_s might contribute to the physiological effects of the MC4R. In addition to the established G_s coupling, observations have, indeed, been made either suggesting direct coupling of the MC4R to other members of the G protein family or the activation of signalling pathways that are insensitive to specific inhibitors of the G_s /PKA pathway. Newman et al., reported that NDP-MSH increases intracellular calcium concentrations in murine, immortalized hypothalamic neurons (GT1-1 cells) endogenously expressing MC4R (Newman et al., 2006). This calcium response was blocked by the MC4R specific antagonist SHU-9119 and sensitive to a specific phospholipase C (PLC) inhibitor. Thus, as suggested by the authors, in GT1-1 cells MC4R might be able to activate the G_q /PLC/calcium pathway. However, in closely related GT1-7 cells, no

melanocortin-induced activation of this pathway was observed (Buch et al., 2009). Mountjoy et al., (Mountjoy et al., 2001) and Nickolls et al., (Simon et al., 2009) reported calcium transients in HEK293 cells recombinantly overexpressing the MC4R, thus confirming the results obtained in GT1-1 cells. However, in these cells melanocortin-induced calcium signals were blocked by the G_{α_s} specific inhibitor, *cholera* toxin (CTX), and most probably not due to the activation of G_q proteins. These data suggest that MC4R-mediated calcium signalling strongly depends on the cellular context.

Considering melanocortin-induced activation of ERK-1/2, a similar picture arises. In HEK293 cells melanocortin-mediated activation of ERK-1/2 was sensitive to the $G_{\alpha_{i/o}}$ specific inhibitor *pertussis* toxin (PTX) (Chai et al., 2006), whereas in GT1-1 or GT1-7 cells ERK-1/2 activation induced by melanocortins was resistant to PTX (Buch et al., 2009; Chai et al., 2006). These observations indicate engagement of the MC4R with PTX-sensitive G proteins. However, it seems that the propensity of this receptor to activate PTX-sensitive G proteins depends on the cell line used. Melanocortin-induced activation of ERK-1/2 is of particular interest, since Sutton et al., reported that a specific ERK-1/2-inhibitor completely blocked anorexigenic effects of the MC4R-specific agonist MTII in rats (Sutton et al., 2005). Given the importance of PKA and ERK-1/2 signalling for the anorexigenic effects of melanocortins, one might assume that the G_s /PKA pathway directly increases ERK-1/2 activity, as shown for other GPCRs (Vossler et al., 1997; Wan and Huang, 1998). Indeed, in the above mentioned study of Sutton et al, MTII-induced activation of ERK-1/2 was abolished by the cAMP inhibitor Rp-cAMPs. However, other studies reported that in GT1-1 cells melanocortin-induced activation of ERK-1/2 does not require PKA but PKC activity and the increase of intracellular calcium concentrations (Chai et al., 2006). Similarly, in MC4R

overexpressing CHO-K1 cells, melanocortin-induced ERK-1/2 activation was insensitive to PKA inhibitors but sensitive to PI3K blockade and thus most probably conveyed through inositol trisphosphate (Vongs et al., 2004). Thus, it remains unclear whether PKA and ERK-1/2 belong to the same or diverging pathways and which signalling components associate the MC4R with these kinases.

Considering recently discovered signalling pathways of GPCR that are not mediated by G proteins but initiated by adapter proteins like arrestins, this scenario becomes even more complex: initially, arrestins have been shown to terminate G protein-dependent signalling after agonist binding to the receptor. However, recent work from many outstanding laboratories has been shown that arrestins also activate cellular effectors like ERK-1/2 in a G protein-independent manner (Shenoy and Lefkowitz, 2005). Thus, MC4R-mediated ERK-1/2 activation could also be explained by this new aspect of GPCR signalling. This is of particular interest, since data from our laboratory suggest that α -MSH and AGRP induce interactions between the MC4R and arrestins in HEK293 or Cos-1 cells (Breit et al., 2006). Thus, arrestin-mediated ERK-1/2 activation by the MC4R could also contribute to melanocortin signalling.

Differences observed in the sensitivity of melanocortin-induced ERK-1/2 signalling to PTX in GT1-1 and GT1-7 cells on the one hand and HEK293 cells on otherhand, suggest that the MC4R couples to members of the $G_{i/o}$ family only when overexpressed in HEK293 cells. However, again the situation appears much more complex, since recently it has been reported that although α -MSH-induced activation of ERK-1/2 signalling in GT1-7 cells is not sensitive to PTX, the toxin affects α -MSH-induced accumulation of cAMP and incorporation of $GTP\gamma S^{35}$ (Buch et al., 2009). Thus, similar to over expressing HEK293 cells, the MC4R has the potential to functionally interact with $G_{i/o}$ proteins,

when endogenously expressed in GT1-7 cells.

The D90N mutation of the MC4R has been associated with severe early-onset obesity. This MC4R variant binds melanocortins with unchanged high affinity, but agonist binding does not initiate G_s signalling. Thus, in the context of alternative G protein coupling the D90N variant of the MC4R represents an excellent tool to analyze putative G_s -independent signalling pathways and to identify structures of the MC4R that are responsible for the coupling to distinct G proteins. Indeed, one study from our laboratory revealed that although this mutant is deficient in any detectable G_s signalling, it induced incorporation of $GTP\gamma S^{35}$ in PTX-sensitive G proteins and decreases basal concentrations of cAMP, indicating that the MC4R-D90N selectively activates $G_{i/o}$ (Buch et al., 2009). This finding strengthens the idea of dual coupling of the MC4R to G_s and $G_{i/o}$ proteins and provides new insights into the molecular mechanism of MC4R-mediated G protein activation.

It has been proposed that receptors exist in an inactive, G protein-uncoupled (R) and an active, G protein-coupled (R^*) conformation (Samama et al., 1993). Over the last decade, it has been debated whether GPCR form only one or more R^* s (Galandrin et al., 2007; Kenakin, 2007). Data obtained for the D90N mutant indicate that a single point mutation in the coding sequence of the MC4R allows for specific activation of $G_{i/o}$ but not of G_s . Hence, one may postulate that the MC4R exists in R^* s specific for G_s or $G_{i/o}$ proteins, indicating that MC4R-propagated signalling is best described in a model consistent with multiple R^* conformations. A multi-state-model of receptor activation raises the intriguing possibility that distinct ligands might be able to differentially stabilize distinct R^* s and therefore selectively activate a particular signalling pathway (ligand-directed signalling). Such so-called biased agonists have recently been identified for members of other GPCR families (Galandrin et al., 2008; Leduc et

al., 2009; Michel and Alewijnse, 2007; Ramachandran et al., 2009; Reversi et al., 2005; Schonbrunn, 2008) and have also been suggested for the MC4R by Nickolls et al.. This work reported that when considering cAMP levels, calcium signals and, in addition, agonist-dependent receptor endocytosis induced by non-peptide versus peptide MC4R agonists, agonist-specific receptor conformations exist that translate agonist binding into different efficacies to activate a given pathway (Nickolls et al., 2005).

In sum, it appears that the MC4R is able to functionally engage with other G proteins than G_s , most probably $G_{i/o}$ and/or G_q (see Tab. 2), and that different active receptor conformations might be responsible for this alternative G protein coupling. However, the ability of the MC4R protein to create these distinct conformations might depend on the cellular context and/or the expression level of the receptor.

1.4 AGRP-induced signalling pathways of the MC4R

In contrast to all other hormone systems known so far, the melanocortin system is the only one that is not just regulated by agonistic peptides but also by two endogenously occurring neuropeptides that block ligand-induced MCR signalling: agouti and “agouti-related protein” (AGRP). Agouti binds to almost all MCR with high affinity, whereas AGRP exhibits rather selective binding towards the MC3R and MC4R (Ollmann et al., 1997) (Tab. 1).

Since binding of AGRP prevents or displaces the binding of melanocortins to the MC3R or MC4R, it has been defined as a classic competitive antagonist (Ollmann et al., 1997). In line with these inhibitory effects on melanocortin signalling, AGRP has been shown to oppose the biological activity of melanocortins *in vivo* and therefore represents an orexigenic stimulus (Bewick et al., 2005; Dhillo et al., 2003; Graham et al., 1997; Luquet et al., 2005; Shutter et al., 1997). This model is based,

for example, on the observation that AGRP-deficiency leads to a lean phenotype of mice and an extended lifespan, when the animals received a high fat diet (Redmann and Argyropoulos, 2006; Wortley et al., 2005). Furthermore, AGRP polymorphisms are associated with resistance to adipositas and development of type-2 diabetes in humans (Argyropoulos et al., 2003; Dubern et al., 2001; Vink et al., 2001).

However, the molecular events responsible for AGRP-mediated physiological effects are not completely understood. Based on the common model of competitive antagonism, effects of AGRP on appetite control depend strictly on the action of melanocortins. Contrasting this assumption, recent studies revealed that the effects of AGRP on appetite control are independent of melanocortin signalling. For example, one study reported that intracerebroventricular injection of AGRP in POMC- and thus melanocortin-deficient mice induces long-lasting increased food uptake (Tolle and Low, 2008). Similarly, another study reported that ablation of AGRP-expressing neurons in the arcuate nucleus leads to starvation not only in wild-type but also in mice that have chronic blockade of melanocortin signalling (Wu et al., 2008). Absence of melanocortin signalling did not even ameliorate the extent of starvation. Both studies provide *in vivo* data indicating that AGRP impairs appetite control in a melanocortin-independent manner, suggesting that mechanisms other than competitive antagonism of MC4R signalling have also to be considered when discussing the physiological effects of AGRP on the molecular level.

One forward explanation for AGRP-mediated actions in the absence of melanocortins is that, in addition to its antagonistic effects, AGRP decreases melanocortin-independent MC4R signalling and thus basal intracellular cAMP concentrations in MC4R expressing cells (Breit et al., 2006; Khong et al., 2001; Nijenhuis et al., 2001; Yang et al., 1999). These actions of AGRP on basal cAMP

production define this neuropeptide as an inverse agonist of the MC4R and could account for the melanocortin-independent effects described above. However, since the importance of basal, ligand-independent MC4R activity in the regulation of energy homeostasis is not clear, it is hard to predict whether or not blocking basal MC4R activity due to the inverse agonistic actions of AGRP can sufficiently account for the melanocortin-independent effects of AGRP observed *in vivo*.

In light of the above mentioned possibility that MC4R are functional engaged with $G_{i/o}$ proteins, it is tempting to speculate that members of this G protein family might also be involved in the effects of AGRP in the absence of melanocortins. Indeed, a recent publication of our laboratory provided data indicating that AGRP is able to induce the incorporation of $GTP\gamma S^{35}$ in PTX-sensitive G proteins in membranes derived from MC4R over-expressing HEK293 cells (Buch et al., 2009). However, in the same cells PTX was not able to block AGRP-mediated reduction of forskolin-induced cAMP accumulation. This confusing observation might be best explained by the activation of G_z proteins that have been described as the only member of the G protein family that decreases cAMP accumulation in a PTX-insensitive manner, but so far proof of an additional coupling of the MC4R to G_z proteins was not successful.

Together, with the discrepancy observed between HEK293 and GT1-7 cells in melanocortin-induced ERK-1/2 activation, we suggest that MC4R signalling in overexpressing HEK293 cells differs dramatically from the signalling observed in GT1-1 or GT1-7 cells (Tab. 3), and that AGRP-mediated signalling in HEK293 cells cannot fully be described by currently known signalling pathways. However, in GT1-7 cells the picture of AGRP-induced signalling appears to be a bit clearer. AGRP-induced incorporation of $GTP\gamma S^{35}$ in PTX-sensitive G proteins was also detectable in this cell line (Buch et al., 2009), indicating that AGRP has the

potential to activate members of the $G_{i/o}$ family via MC4R in endogenous expression systems. In striking contrast to HEK293 cells, AGRP did not decrease but increased forskolin-induced cAMP production in GT1-7 cells (Buch et al., 2009). At first glance, this behavior of AGRP appears to be paradox. However, it should be taken into account that AC isoforms can be grouped into 3 subfamilies. Members of group 2 have been shown to be less sensitive to $G_{i/o}$ -mediated inhibition, but are sensitized by $\beta\gamma$ -subunits released from $G_{i/o}$. Indeed, transcripts of AC2 and AC4, which belong to group 2, were detectable in GT1-7 cells (Buch et al., 2009) and, thus, AGRP-mediated activation of $G_{i/o}$ -signalling might increase forskolin-induced cAMP production by sensitization of group 2 AC isoforms. This hypothesis was firstly supported by the blocking effects of PTX on AGRP-mediated enhancement on forskolin-induced cAMP accumulation and secondly by the inhibitory effects of the recently described cell-permeable $\beta_1\gamma_2$ -subunit inhibitor gallein on AGRP-induced forskolin-promoted cAMP accumulation (Buch et al., 2009). Thus, it appears that AGRP activates PTX-sensitive G proteins to release $\beta\gamma$ -subunits that sensitize AC in GT1-7 cells.

In conclusion, besides its antagonistic and inverse agonistic actions, AGRP has the propensity to activate PTX-sensitive G proteins in GT1-7 cells and, thus, to behave like a biased agonist of the MC4R that is able to selectively activate $G_{i/o}$ and to simultaneously block G_s signalling. We believe that in overexpressing systems (like HEK293 cells) this biased agonism affects distinct down-stream effectors of MC4R differently when compared to GT1-7 cells (see Tab. 3). Together with our results obtained for the MC4R-D90N mutant we suggest a multi-state-model of MC4R activation that rests on the extended version of the allosteric ternary complex model of receptor activation (Samama et al., 1993). This model implies at least one additional active receptor state (R^{**}) that binds $G_{i/o}$ proteins with higher affinity and MSH with

lower affinity when compared to R* (for details see Fig. 2). Biased agonistic actions of AGRP could have strong implications on MC4R-induced regulation of energy homeostasis. However, *in vivo* data considering this aspect are not established yet. Noteworthy, a recent publication described that melanocortins modulate the excitability of hypothalamic neurons by alterations of resting potassium conductance (Fu and van den Pol, 2008). Interestingly, inward-rectifier potassium channels ($K_{IR3.x}$) are regulated by $\beta\gamma$ -subunits released from $G\alpha_{i/o}$ (Leaney, 2003; Milovic et al., 2004). In agreement with biased agonistic actions of AGRP, the authors observed inhibition of these hypothalamic neurons by AGRP in a PTX-sensitive manner. Due to the exclusive G_s coupling of the MC4R postulated so far, it has been contended by the authors that PTX-sensitive signalling of AGRP is most probably mediated by another, yet unidentified GPCR. Based on our new model, it will be enlightening to clarify whether AGRP-mediated PTX-sensitive inhibition of hypothalamic neurons is blocked by MC4R selective antagonists, indicating that this effect is mediated by MC4R-dependent activation of $G_{i/o}$ proteins.

2 Fine-tuning of MC4R signalling

Although the importance of MC4R signalling in the regulation of energy homeostasis is well established, surprisingly little is known about the molecular events responsible for this physiological role. Furthermore, some studies obtained contradictory results (often in different cell models) and alternative G protein coupling and inverse or biased agonism additionally complicate the picture. Thus, in the following, we will discuss aspects of receptor signalling that are known to fine-tune GPCR signalling and whose considerations might contribute to the understanding of MC4R-induced signalling.

2.1 GPCR activity-modifying accessory proteins

For a long time signalling of GPCR has been described as a unidirectional process simply regulated by the affinity of the receptor to its cognate ligand and G protein. In recent years fundamental studies delineated a pluridimensional picture of GPCR signalling, since influence of receptor multimerisation and accessory proteins has been revealed. Interactions among GPCR might appear as homo- or heteromultimerisation. The latter affects many basic functions of GPCR signalling such as ligand binding and G protein coupling (Angers et al., 2002; Milligan et al., 2006). For the MC4R heteromultimerisation with the MC3R and the G protein-coupled receptor-7 (GPR7), which are both expressed in similar hypothalamic regions compared to the MC4R, has been observed (Biebermann et al., 2003; Elsner et al., 2006; Rediger et al., 2009). The impact of these interactions on melanocortin signalling is still unsettled, therefore, further analysis of MC4R containing receptor complexes might contribute to a better understanding of melanocortin and AGRP signalling.

The concept of accessory proteins with single transmembrane domains that regulate GPCR function has been put forward by the observation that the expression of the 3 different RAMPs (receptor-activity modifying protein) proteins (RAMP1-3) dramatically affects cell surface expression and pharmacological properties of two distinct receptors (Sexton et al., 2001). RAMPs are membrane-spanning proteins with a single transmembrane domain that exhibit no ligand-binding or signalling properties by themselves. However, co-expression of RAMP-1 or -3 with the calcitonin receptor creates the receptor for amylin, a hormone whose blood level increases similarly to that of insulin after meal uptake (Armour et al., 1999; Muff et al., 1999; Tilakaratne et al., 2000; Zumpe et al., 2000). The calcitonin-receptor-like receptor expressed without any RAMPs

remains in the ER, but co-expression with RAMP-2 or -3 gives rise to the adreno-medullin receptor and with RAMP-1 to the calcitonin gene-related receptor on the cell surface (Buhlmann et al., 1999; Christopoulos et al., 1999; Ono et al., 2000).

In the case of the MC4R three families of different accessory proteins might be of interest: syndecans, mahoganoid and MRAPs (melanocortin receptor accessory proteins).

2.1.1 Syndecans

Syndecans are a group (syndecan-1 to -4) of heparan-sulfate proteoglycans that exist in a membrane-bound and in a soluble, shedded form. Membrane-bound syndecans act as co-receptors and are involved in several physiological processes (Reizes et al., 2006). For example, mice that transgenetically overexpress syndecan-1 in the hypothalamus have severe maturity-onset obesity and diabetes mellitus type 2. Targeted disruption of the syndecan-3 gene leads to resistance of the mice to diet-induced obesity (Reizes et al., 2001). A positive association of obesity and syndecan-3 polymorphisms was also found in humans (Ha et al., 2006). Interestingly, it has been shown in various mouse models that syndecans enhance AGRP-induced phenotypes and suggested that syndecans enhance the blocking effects of AGRP on MC4R-signalling (Reizes et al., 2003). Although the exact mechanisms regulating the interaction between AGRP and syndecans are not clear yet, it has been put forward that the membrane-bound form of syndecans co-localizes with the MC4R and binds AGRP. This syndecan/AGRP complex could decrease the affinity of melanocortins to the MC4R and thus inhibit melanocortin signalling (Reizes et al., 2006). This model has been supported by the observation that co-expression of MC4R and syndecan-3 in HEK293 cells enhances the inhibitory effects of AGRP on melanocortin-induced cAMP accumulation (Reizes et al., 2001). However, another

study noticed that the domains of AGRP responsible for the binding to syndecan-3 are cleaved from the peptide before it leaves the cell, suggesting that the influence of syndecan-3 on food intake is independent from its interactions with AGRP and/or the MC4R (Creemers et al., 2006).

2.1.2 Mahoganoid

The mouse mahogany protein is the orthologue of the human attractin and represents a receptor involved in suppression of obesity and skin pigmentation (Gunn et al., 1999; Nagle et al., 1999). Since the effects of mahogany observed in various mouse models are mainly mediated by agouti and not by AGRP, they are not in the focus of this review. However, the mouse mahoganoid, also called mahogunin ring finger-1 (MGRN-1) protein, and its human orthologue (KIAA0544) are cytosolic ring domain-containing ubiquitin ligases that effect, similarly to mahogany, energy balance and pigmentation (Phan et al., 2002). Mouse models suggest that the MC4R as well as AGRP might contribute to these effects. On the molecular level three models have been suggested to describe the biological effects of mahoganoid 1) mahoganoid increases expression or activity of AGRP; 2) influences the physical proximity of AGRP to the MC4R or 3) decreases the availability of melanocortins for the MC4R (Phan et al., 2002). A more recent study analyzed the effects of mahoganoid on MC4R-induced cAMP production in HEK293 cells (Perez-Oliva et al., 2009). These experiments revealed that mahoganoid decreased MC4R signalling without affecting other G_s-coupled receptors. The inhibitory effects of mahoganoid were independent of receptor surface expression, ubiquitination, internalization or protein stability and occurred upstream of G_s coupling and AC activation. Therefore, a competition of mahoganoid and G_s proteins for the MC4R

might be a straightforward explanation for these effects, as suggested by the authors.

2.1.3 Melanocortin receptor accessory proteins (MRAPs)

Like RAMPs, MRAPs are proteins with one transmembrane domain that have recently been described to modify MCR signalling (reviewed by (Hinkle and Sebag, 2009; Webb and Clark, 2010)). In most cell types heterologous expression of the MC2R does not yield a functional receptor (Noon et al., 2002). Direct interactions of MRAPs with the MC2R are required for the receptor to traffic from the ER to the cell surface (Metherell et al., 2005). Recently, it was described that MRAP and MRAP-2 also interact with the four remaining MCR (Chan et al., 2009). In contrast to the benefit the MC2R experiences from its interaction with MRAPs, all other MCR suffer from the co-expression with MRAPs (Sebag and Hinkle, 2009). The MC5R for example, which is well translocated from the ER to the cell surface when expressed alone, is trapped inside when co-expressed with MRAPs. Similarly but maybe to a lesser extent, MRAPs decrease the translocation of the MC4R to the plasma membrane and inhibit its efficacy to stimulate cAMP accumulation. However, to date it is not clear if the effects of MRAPs on MC4R-mediated cAMP accumulation solely result from reduced cell surface expression or if MRAPs are also able to travel with the MC4R to the cell surface and influence there its coupling to G proteins and, thus, MC4R signalling.

2.2 Organization of MC4R signalling in specialized membrane microdomains (lipid rafts)

Lipid rafts are cholesterol- and sphingolipid-rich microdomains in the plasma membrane (Helms and Zurzolo, 2004; Simons and Toomre, 2000). The main aspect of lipid rafts is to regulate the activity of a given pathway by their

propensity to include or exclude signalling components in the signalling cascade. Organization of GPCR signalling by lipid rafts has been reported for the cAMP and calcium pathway numerous times (Helms and Zurzolo, 2004; Simons and Toomre, 2000).

Considering in particular cAMP signalling, it has been shown that chemically induced cholesterol depletion of the plasma membrane (resulting in the disruption of lipid rafts) increases cAMP levels, because lipid rafts separate various components of the G_s /AC-pathway from each other and, thus, exert a tonic inhibition on cAMP production (Ostrom et al., 2001; Ostrom et al., 2000; Pontier et al., 2008). Most AC isoforms are found in lipid rafts (Liu et al., 2008; Thangavel et al., 2009) while G_{α_s} and G_{α_o} subunits are mostly found in non-lipid rafts areas (Abankwa and Vogel, 2007; Ostrom et al., 2001; Pontier et al., 2008). Interestingly, for the G_{α_o} protein which is highly expressed in neurons, it has been reported that in cerebellar neurons the inactive form co-localizes with $\beta\gamma$ -subunits in non-lipid rafts, but after activation, G_{α_o} leaves its $\beta\gamma$ -subunits behind and translocates into lipid rafts, most probably to regulate AC activity (Yuyama et al., 2007). Interestingly, accumulation of AC subtypes in lipid rafts has mainly been reported for AC3, AC5, AC6 and AC8 (which are all $G_{\alpha_{i/o}}$ -sensitive but insensitive to $\beta\gamma$ -induced sensitization, see Tab. 4) but not for others (Schwencke et al., 1999). Thus, there might be the possibility that $G_{\alpha_{i/o}}$ -sensitive ACs accumulate in lipid rafts while ACs that are sensitized by $\beta\gamma$ -subunits enrich in non-lipid raft areas.

To our knowledge, so far, no data are available about the localization of the MC4R in lipid rafts; however, one study suggests that the closely related MC3R subtype is located in lipid rafts (Wachira et al., 2007). The distribution of lipid rafts over the cell surface, their lipid composition and/or inventory of scaffolding proteins has been shown to strongly depend on the cell type and morphogenesis (Helms and

Zurzolo, 2004; Pontier et al., 2008; Simons and Toomre, 2000). In such a scenario, the number and/or composition of lipid rafts in different cells could affect the regulation of AC activity by $G_{\alpha_{i/o}}$ - and $\beta\gamma$ -subunits. Given the contrasting effects of AGRP on cAMP accumulation in HEK293 and GT1-7 cells, suggested to be mediated by $\beta\gamma$ -subunits in the latter, altered organization of these signal components in microdomains depending on the cell type might explain the observed differences. Similarly, different organization of components of the calcium signalling pathway in lipid rafts of membranes from GT1-1 and GT1-7 cells, could contribute to the differences observed in melanocortin-induced calcium transients. For future perspectives, it might be advantageous to analyze the microdomain localization of the MC4R and its downstream effectors in different cell lines and then to compare melanocortin- and/or AGRP-induced signalling in these cells.

3 Conclusions

Although the important role of the MC4R in decreasing food intake and increasing energy expenditure is widely accepted, pharmacological intervention in MC4R signalling plays only a minor role in the therapy of obesity or anorexia. Thus, screening for drugs that significantly modulate MC4R activity *in vivo* is ongoing work. Besides others, one drawback in this progress is that signalling pathways of the MC4R build up a multi-layered, entangled network: various endogenous ligands activate multiple G proteins with different efficacies; accessory proteins such as syndecans, mahoganoid and MRAPs contribute to the fine-tuning of MC4R function and organisation of signalling components in microdomains and even receptor heteromultimerisation might also be involved. Thus, disentangling the network of MC4R-induced signalling in the particular model system used might ease and improve both drug discovery and academic research.

4 References

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Table 1 Melanocortins and their Receptors

receptor subtype	rank order of potency	antagonists	central effects	peripheral effects
MC1R	α -MSH = β -MSH = ACTH > γ -MSH	agouti	-	pigmentation
MC2R	ACTH	agouti	-	glucocorticoid production
MC3R	α -MSH = β -MSH = ACTH = γ -MSH	agouti and AGRP	control of body weight	release of cytokins
MC4R	α -MSH = β -MSH > ACTH > γ -MSH	agouti and AGRP	control of body weight and appetite regulation of blood pressure	inhibition of inflammation
MC5R	α -MSH = β -MSH > ACTH > γ -MSH	agouti	-	sebum production

Data from two different sources (MacNeil et al., 2002; Schioth et al., 2005) were compiled.

Table 2 Alternative Signalling of Melanocortins

effector	ligand	pathway	cell system	reference
calcium release	α -MSH	CTX but not PTX sensitive: Gs	HEK293 cells	Mountjoij et al.,
calcium release	various, incl. NDP- and α -MSH	-	HEK293 cells	Nickolls et al.,
calcium release	NDP-MSH	sensitive to PLC inhibition: Gq	GT1-1 cells	Newmann et al.,
no calcium release	α -MSH or NDP-MSH	-	GT1-7 cells	Buch et al.,
ERK activation	NDP-MSH	sensitive to PKC and calcium inhibition	GT1-1 cells	Chai et la.,
ERK activation	NDP-MSH	PTX sensitive: Gi/o	HEK293 cells	Chai et la.,
ERK activation	NDP-MSH	not PTX sensitive	GT1-1 cells	Chai et la.,
ERK activation	α -MSH	not PTX sensitive	GT1-7 cells	Buch et al.,
ERK activation	NDP-MSH	insensitive to PKA but sensitive to PI3K inhibition	CHO-K1 cells	Vongs et al.,
ERK activation	NDP-MSH	insensitive to PKA inhibition	HEK293 cells	Chai et la.,
ERK activation	MTII	sensitive to PKA inhibition	<i>in vivo</i>	Sutton et al.,
GTP γ S ³⁵ incorporation and cAMP production	α -MSH	PTX sensitive: Gi/o	HEK293 and GT1-7 cells	Buch et al.,
signalling by Gs deficient mutant	α -MSH	PTX sensitive: Gi/o	HEK293 cells	Buch et al.,

Table 3 Alternative Signalling of AGRP

effector	pathway	cell system	reference
GTPγS³⁵ incorporation	PTX sensitive	HEK293 cells	Buch et al.,
GTPγS³⁵ incorporation	PTX sensitive	GT1-7 cells	Buch et al.,
decrease of FSK-elevated cAMP	PTX insensitive	HEK293 cells	Buch et al.,
decrease of FSK-elevated cAMP	inhibition of G _s	B16/G4F cells	Nijenhuis et al.,
increase of FSK-elevated cAMP	PTX and gallein sensitive	GT1-7 cells	Buch et al.,
ERK activation	PTX sensitive	HEK293 cells	unpublished data
receptor endocytosis	-	HEK293 cells	Breit et al.,
no receptor endocytosis	-	HEK293 cells	Nickolls et al.,
receptor endocytosis	-	GT1-7 cells	Breit et al.,
potassium conductance	PTX sensitive	hypothalamic neurons	Fu et al.,

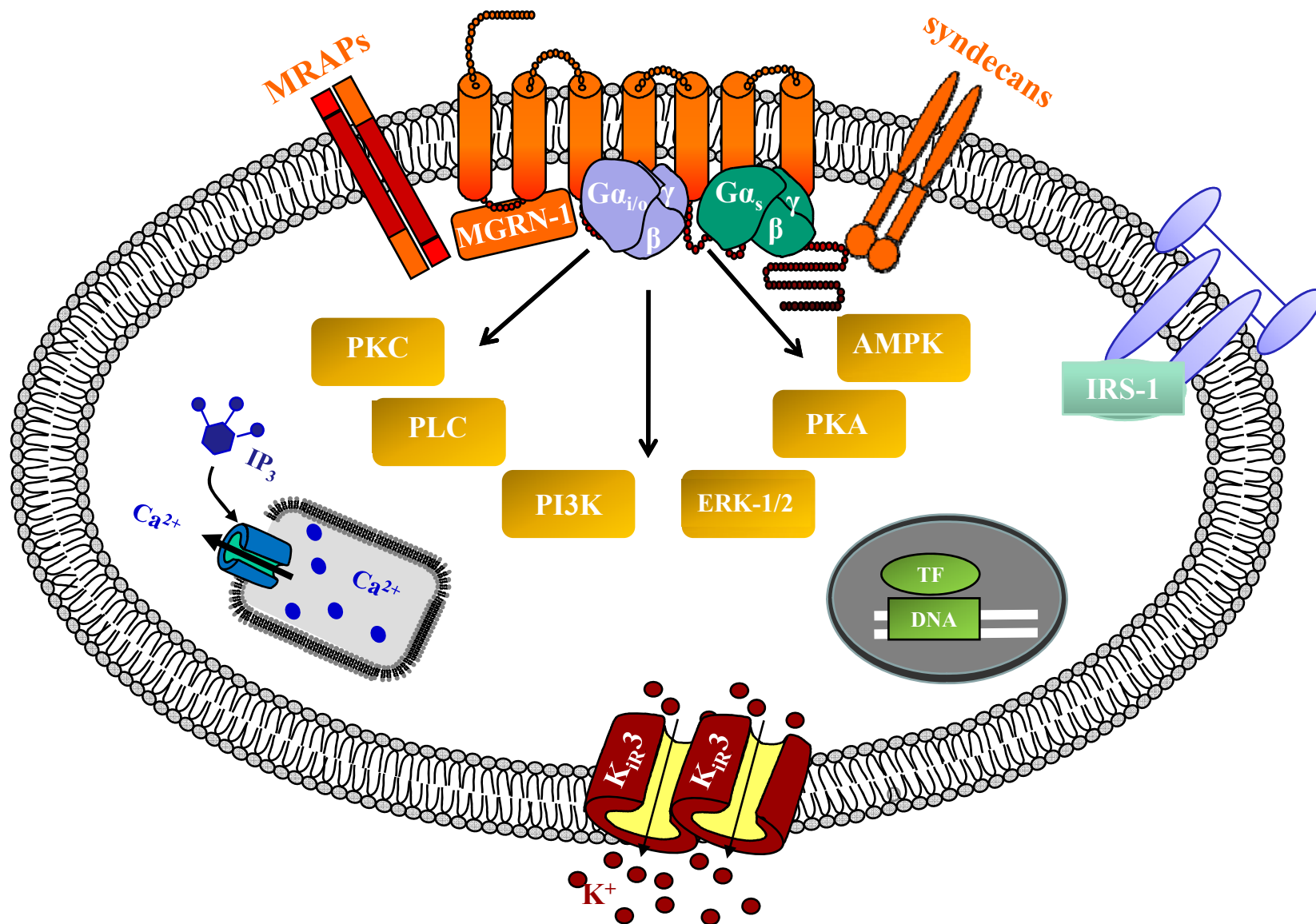


Fig.1: Regulation of MC4R signalling by accessory proteins and alternative G protein coupling leads to the activation of numerous kinases, lipases and, in turn, to the modification of various downstream effectors including transcription factors (TF), ion channels and IRS-1. Data obtained in different studies and various cell models are compiled.

Fig. 1

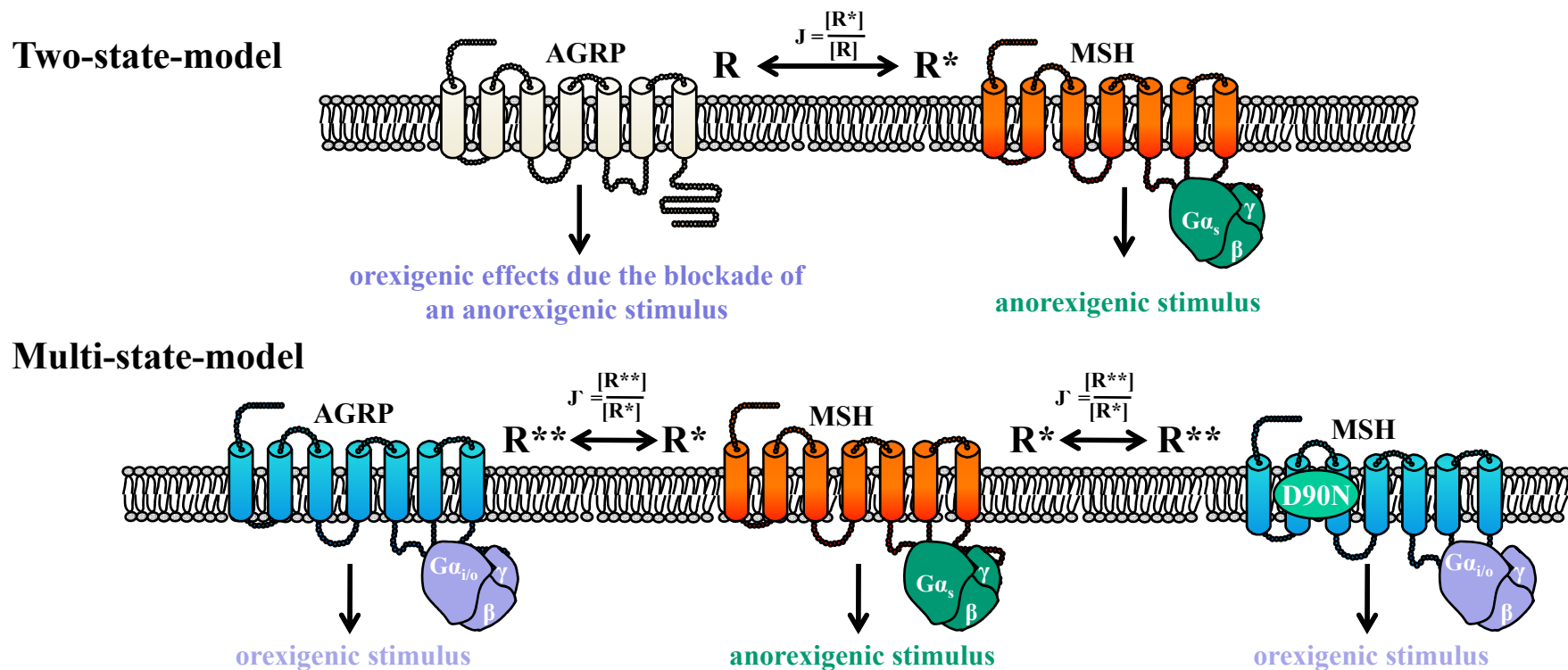


Fig.2 Schemes of MC4R activation that base on the extended version of the allosteric ternary complex model (Samama, 1993)

Upper panel: within a two-state-model AGRP stabilizes the G protein-uncoupled, inactive receptor conformation (R) and thus 1) blocks MSH binding to the G protein-coupled, active form (R*) (competitive antagonism) and 2) decreases basal G_s activation (inverse agonism). Both events contribute to the orexigenic effects of AGRP. Of note, the efficacy of AGRP to decrease basal G_s activation strongly depends on the isomerisation constant J which reflects the ratio of $[R^*]$ over $[R]$ and might be different in various cell models.

Lower panel: within a multi-state-model AGRP stabilizes an active receptor conformation (R**) that differs from R* stabilized by MSH. R** binds $G_{i/o}$ proteins with higher affinity and MSH with lower affinity than R*, thus binding of AGRP to R** has three consequences: 1) displacement of MSH from R* (competitive antagonism), 2) uncoupling of G_s from R* even in the absence of MSH (inverse agonism) and 3) activation of $G_{i/o}$ proteins (biased agonism). The exchange of D to N at position 90 of the MC4R wild-type selectively prevents the mutated protein from building up R* ($J_{\text{mutant}} > J_{\text{wild-type}}$), thus MSH binds preferentially to R** and activates more $G_{i/o}$ - than G_s proteins.

Fig. 2